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# Initial Report of *Inocybe costinitii* in Türkiye with Morphological and Molecular Data

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## ABSTRACT

*Inocybe* specimens were collected from Ankara University Beşevler 10. Yıl Campus, (Ankara, Türkiye) on October 19, 2022. As a result, the samples were identified as *I. costinitii*, a new record for Turkish *Inocybe*. This study presents a detailed description of this newly recorded species, covering aspects such as its location, observations of its habitat, geographical coordinates, the date of collection, and photographs that highlight its macroscopic and microscopic characteristics. Moreover, the study features the species' drawings and some of its microscopic traits. The study is enhanced by images obtained from a scanning electron microscope (SEM), briefly examining the spore features and discussed briefly.

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## Morfolojik ve Moleküler Verilerle *Inocybe costinitii*'nin Türkiye'deki İlk Raporu

## ÖZET

*Inocybe* örnekleri Ankara Üniversitesi Beşevler 10. Yıl Kampüsü'nden (Ankara, Türkiye) 19 Ekim 2022 tarihinde toplanmıştır. Sonuç olarak, bu örnekler Türkiye *Inocybe* cinsi için yeni bir kayıt olan *I. costinitii* olarak tanımlanmıştır. Bu çalışma, yeni kaydedilen bu türün konumu, yaşam alanı gözlemleri, coğrafi koordinatları, toplanma tarihi ve makroskobik ve mikroskobik özelliklerini vurgulayan fotoğraflar gibi hususları kapsayan ayrıntılı bir tanımını sunmaktadır. Araştırmada ayrıca türün bazı mikroskobik özelliklerinin çizimleri de yer almaktadır. Çalışma, spor özelliklerinin derinlemesine incelenmesini sağlayan taramalı elektron mikroskobundan (SEM) elde edilen görüntülerle zenginleştirilmiş ve kısaca tartışılmıştır.

**Mikoloji**

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**Anahtar Kelimeler**
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## INTRODUCTION

Belonging to the family *Inocybaceae*, the genus *Inocybe* is recognized as one of the most diversely populated taxa within the *Agaricales*, comprising around 1050 documented species (Dovana et al., 2023). Recent studies indicate that this genus embarked on a swift and widespread evolutionary journey approximately 52 to 79 million years ago (Fachada et al., 2024). The genus exhibits a broad ecological amplitude, inhabiting a spectrum of climatic zones from tropical to Arctic regions (Altuntaş et al., 2019). Ecologically, *Inocybe* species predominantly colonize many forest habitats, spanning deciduous and coniferous forests (Akata et al., 2023).

Morphologically, *Inocybe* species are identified by their relatively diminutive and subtly hued basidiomata (Kuyper, 1985). The pileus is characterized by its dry texture and may exhibit surface features such as scales, fibrils, or cracks. The lamellae generally present a muted brown hue, complemented by the presence of a stipe. A distinctive feature of the genus members is its unique odor, which plays a crucial role in their identification. The spores bear a muted brown coloration, are encased by somewhat thickened and smooth walls, and manifest in a variety of morphologies including angular, nodulose, or spinose

forms, conspicuously lacking a germ pore and the presence of cystidia, are often adorned with apices embedded with calcium oxalate crystals (Kuyper, 1986; Matheny & Kudzma, 2019; Bandini et al., 2020; 2021).

Fachada et al. (2024) estimate that the genus exhibits substantial diversity, with a global species count ranging from 3,000 to 5,000. Akata et al. (2023) state that, thus far, 92 *Inocybe* species have been reported from Turkey. Among them, 11 species have been reclassified into different genera: 7 have been transferred to *Inosperma* (Kühner) Matheny & Esteve-Rav., and 4 to *Mallocybe* (Kuyper) Matheny, Vizzini & Esteve-Rav. Due to recent updates, the count of *Inocybe* species recorded in Turkey has been revised to 81 (Akata et al., 2023; Matheny et al., 2020).

The objective of the present study is to contribute to the mycobiota of Turkey.

## MATERIALS and METHODS

This study utilized a comprehensive approach, combining traditional and cutting-edge molecular methods to analyze and categorize samples collected from Beşevler 10. Yıl Campus of Ankara University in Ankara, Türkiye. It involved thoroughly examining both the samples' macroscopic and microscopic attributes, enhanced by studying ribosomal DNA (rDNA) sequences using Internal Transcribed Spacer (ITS) sequencing techniques.

### Morphological Characterization

*Inocybe* samples were collected from the study area, and a comprehensive evaluation of their macroscopic and environmental attributes was conducted in their natural habitat. Subsequently, these specimens were subjected to microscopic examination in a controlled laboratory environment. A Euromex Oxion Trinocular light microscope facilitated the observation of the specimens' fine details at a magnification of 100X. To ensure the precision of the observations, each microscopic characteristic was quantified approximately 30 times. Some chemical agents, including 5% potassium hydroxide (KOH), and Congo red, were employed for the analysis process. The collected data from these measurements underwent statistical analysis. For scan electron microscope (SEM) analysis, small fragments of the specimens were affixed to stubs using double-sided adhesive tape and sputter-coated with gold. These prepared samples were then analyzed using an EVO 40XVP SEM, produced by LEO Ltd. in Cambridge, UK, with the device operating at an accelerating voltage of 20 kV.

The methodologies for morphologically identifying the samples were based on the protocols described in research studies by Bizio et al. (2016) and Bandini et al. (2021). Following their identification, the samples were preserved at the Fungarium of Ankara University, located within the Faculty of Science, Biology Department.

### Molecular Characterization

#### *Determination of the ITS rDNA sequences*

The genomic DNA of the samples, identified as ANK AKATA 8687, was extracted employing the CTAB method, following the protocol described by Rogers & Bendich (1994). The quality and quantity of the extracted DNA were assessed using a Nanodrop Lite Thermo Scientific spectrophotometer. This DNA then served as the template for the PCR amplification of the Internal Transcribed Spacer (ITS) rDNA regions, using the ITS1 forward (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 reverse (5'-TCCTCCGCTTATTGATATGC-3') universal primers, according to the approach outlined by Martin & Rygiewicz (2005). The PCR amplification products were subjected to agarose gel electrophoresis, followed by purification with the Expin Gel PCR and CleanUp SV Kit (GeneAll). For Sanger dideoxy sequencing, the purified samples were processed using the BigDye™ Direct Cycle Sequencing Kit (Thermo Fisher Scientific) with the ITS1 and ITS4 primers. The sequenced PCR products were then analyzed on an ABI Prism 3130 Genetic Analyzer. The methodologies for agarose gel electrophoresis and Sanger sequencing were implemented by the protocols provided by Chen et al. (2014).

#### *Molecular Phylogeny Study*

The sequencing data derived from the ITS1 and ITS4 primers were compiled using the Clustal Omega online sequence alignment tool (https://www.ebi.ac.uk/jdispatcher/msa/clustalo). This compiled data was subjected to a BLASTn search to identify its similarity index. Based on the BLAST search results, sequences corresponding to the target group (in-group) and a reference group (out-group) were sourced from the NCBI GenBank database. These sequences were aligned with the compiled ones using the ClustalW algorithm within the MEGAX software, following the method outlined by Kumar et al. (2018). This alignment served as the foundation for subsequent phylogenetic analysis. The evolutionary relationships of the sample series ANK AKATA 8687 were inferred using the Maximum Likelihood method, employing the T92+G model for nucleotide substitution as introduced by Tamura (1992). The accuracy of this phylogenetic assessment was improved by using a bootstrap technique with 1000 repetitions (Felsenstein, 1985). The resulting phylogenetic tree provides insights into the evolutionary relationships among the samples, placing them within the broader framework of fungal classification.

## RESULTS

The recent report of a newly recorded species is meticulously documented, highlighting critical data, such as the collection date, precise location, environmental habitat, and geographical coordinates, alongside the unique collection numbers. The description covers the species' macroscopic and microscopic traits, providing a thorough understanding of its morphology. Moreover, the utilization of scanning electron microscopy (SEM) to capture images of the spores presents an in-depth look at the intricate features that define this species.

### Taxonomic overview

*Fungi*

*Basidiomycota* R.T. Moore

*Agaricales* Underw.

*Inocybe costinitii* Bizio, Ferisin & Dovana (2016), (Figure1-6).

Bizio et al. (2016) provided an in-depth examination of type collections.

Figure 1. Fruit bodies of *Inocybe costinitii*.
*Şekil 1. Inocybe costinitii'nin fruktifikasyonları.*

### Macroscopic and microscopic features

**Pileus** 20–30 mm diam., campanulate, occasionally featuring a wide, blunt umbo. Initially, the margin is inflexed, becoming erect and frequently displaying fissures. **Surface** smooth, fibrous texture with minute, radially arranged scales, displaying a color gradient from grayish brown to pale brown, with the center being lighter, uniformly enveloped by a delicate, white veil persisting on the surface for an extended duration. **Lamellae** sparsely distributed, and adnate, transitioning in color from beige grey to pale brownish, ultimately turning brown, edges eroded and exhibit a whitish hue. **Stipe** 30–40 × 5–6 mm, solid, tapering to cylindrical towards the base, culminating in a subtly margined, diminutive bulb, apex pruinose, with its coloration being predominantly white, albeit with a slight brownish tint near the base, and longitudinally striated. **Flesh** firm and whitish. **Odor** spermatic. **Taste** non-distinctive. **Spores** (9.6–) 10–11.3 (–12) × (5.7–) 6–7 (–7.3) µm, Q = (1.50–) 1.55–1.74 (–1.80), Qav = 1.64, smooth, yellow-brown to brownish, amygdaliform to sub-amygdaliform, featuring a conical to slightly papillate apex. **Basidia** 34–41 × 12–16 µm, hyaline, clavate, typically with four, but occasionally two sterigmata. **Pleurocystidia** 55–74 × 18–24 µm, characterized by a thick-walled composition, changing color to a yellowish or pale yellow-green when exposed to 5% potassium hydroxide (KOH), predominantly fusiform or subfusiform, occasionally clavate or somewhat lageniform or subcylindrical, often with an extended peduncle, either small or large calcium oxalate crystals at the apex.

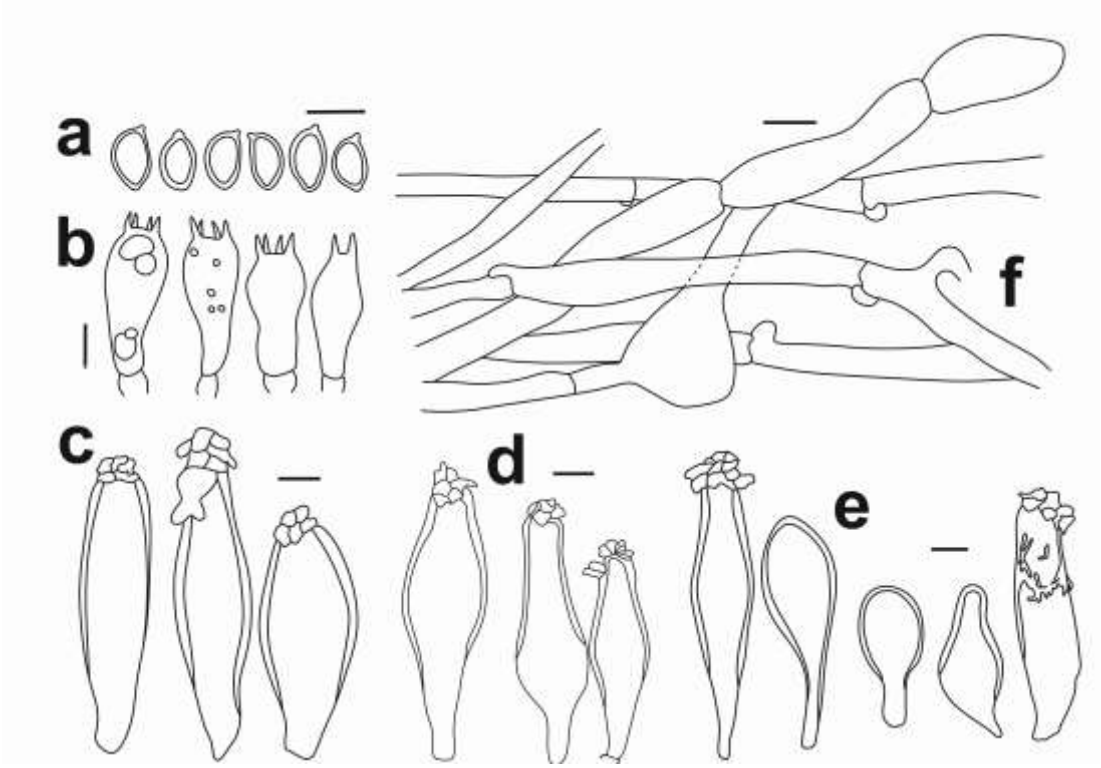


Figure 2. *Inocybe costinitii* : **a.** spores**, b.** basidia**, c.** pleurocystidia**, d.** cheilocystidia, **e.** caulocystidia, **f.** pileipellis (scale bars: 10 µm).

*Şekil 2. Inocybe costinitii: a. sporlar, b. basidyumlar, c. pleurosistidyumlar, d. keylolosistidyumlar, e. kaulosistidyumlar, f. pileipellis (ölçek:10 µm).*

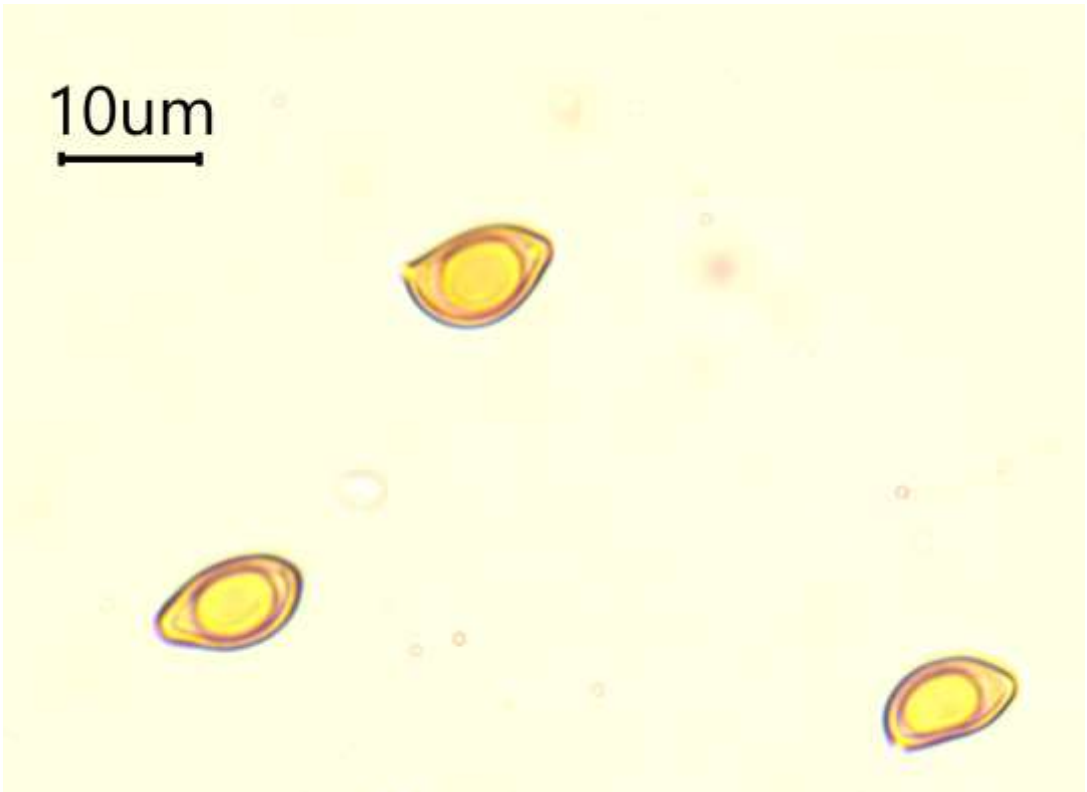


Figure 3. Spores of *Inocybe costinitii* (LM, in KOH).

*Şekil 3. Inocybe costinitii'nin sporları (LM, KOH'ta).*

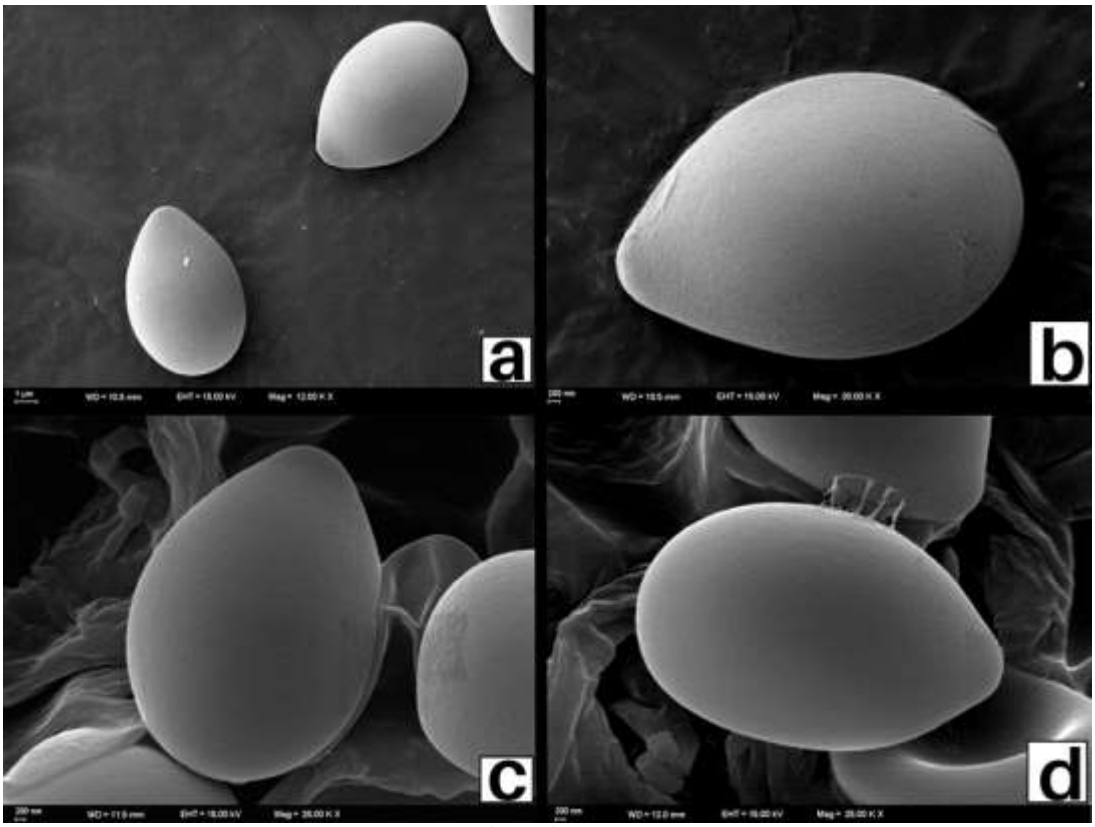


Figure 4. *Inocybe costinitii:* a-d. spores (SEM).
*Şekil 4. Inocybe costinitii: a-d. sporlar (SEM).*

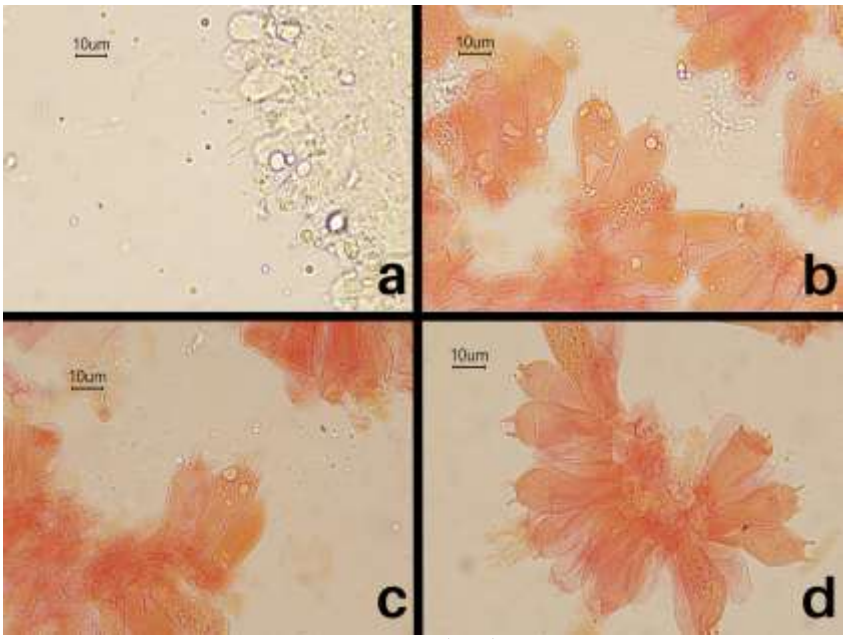


Figure 5. Basidia of *Inocybe costinitii* (LM): a. in KOH, b-d. in Congo red.
*Şekil 5. Inocybe costinitii'nin basidyumları (LM): a. KOH'ta, b-d. Kongo kırmızı'sında.*

**Cheilocystidia** 48–62 × 12–20 µm, similar to pleurocystidia but marginally shorter and thicker. **Caulocystidia** 35–75 × 14–22 µm, clavate to fusiform, and occasionally ventricose, some smooth, while others with crystalline formations at the apex, rarely urticoid-like structures observed, found mainly at the apex of the stipe. **Pileipellis** 5–14 µm broad, thick-walled, consisting of periclinal hyphae, clamp connections present in all tissues.

**Material examined:** TÜRKİYE— Ankara, Ankara University Beşevler 10. Yıl Campus, under pine, 39° 56' N, 32° 50' E, 860 m, 19.10.2022, ANK AKATA 8687. (nrITS rDNA sequence GenBank accession number: PP494209.1).

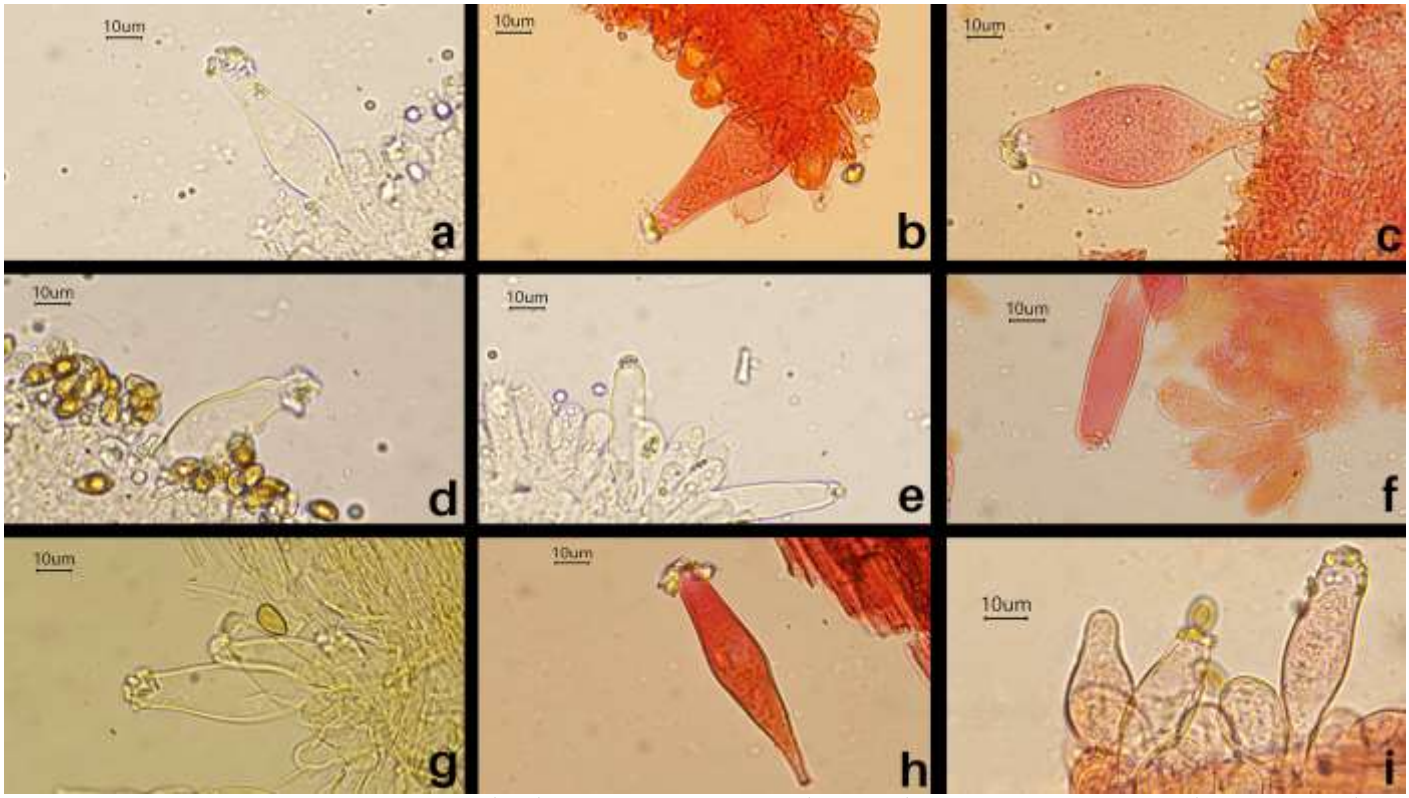


Figure 6. Cystidia of *Inocybe costinitii* (LM): a. pleurocystidium (in KOH), b,c. pleurocystidium (in Congo red), d. cheilocystidium (in KOH), e. cheilocystidia (in KOH), f. cheilocystidium (in Congo red), g. caulocystidium (in KOH), h. caulocystidium (in Congo red), i. caulocystidia (in Congo red).

*Şekil 6. Inocybe costinitii (LM) sistidyumları: a. pleurosistidyum (KOH'ta), b,c. pleurosistidyumlar (Kongo kırmızısı'nda), d. keylosistidyum (KOH'ta), e. keylosistidyum (KOH'ta), f. keylosistidyum (Kongo kırmızısı'nda), g. kaulosistidyum (KOH'ta), h. kaulosistidyum (Kongo kırmızısı'nda), i. kaulosistidyumlar (Kongo kırmızısı'nda).*

### Evolutionary History of ANK AKATA 8687

The evolutionary lineage of the specimen ANK Akata 8687 was explored based on its nrITS rDNA sequence, obtained using standard molecular techniques and archived in the NCBI GenBank under the accession number PP494209.1. To investigate its evolutionary connections, nrITS rDNA sequences from various *Inocybe* genus members were chosen for comparison, with the nrITS rDNA sequence of *Peziza montirivicola* serving as an outgroup. Molecular phylogenetic analysis revealed nine distinct clades, including Clade 7, consisting of the isolates of *Inocybe costinitii* (KX686581.1, PP794435.1) and ANK Akata 8687. Other clades (Clades 1-6 and Clades 8-9) comprised different *Inocybe* species. *Peziza montirivicola* formed a separate branch, indicating its outgroup status. BLAST analyses revealed a similarity of over 99% between the nuclear ITS rDNA sequences of ANK Akata 8687 and a single isolate of *I. costinitii* (Bizio et al., 2016). Phylogenetic analyses affirmed the close relationship between ANK Akata 8687 and *I. costinitii*, with a bootstrap value of 100%, indicating the reliability of their grouping.

## DISCUSSION and CONCLUSION

The genetic diversity of fungal species far exceeds their morphological diversity, prompting the integration of genetic information with traditional morphological methods for more accurate species identification. Various genetic markers, including rRNA gene regions such as nrITS, nrSSU, and nrLSU, along with sequences of protein-coding genes, have been employed in molecular systematic studies for decades (Raja et al., 2017). ITS is widely used in fungal molecular taxonomy among these markers, offering valuable insights (White et al., 1990; Akata & Erdoğdu, 2020; Akata et al., 2024; Altuntaş et al., 2021) Furthermore, advancements in high throughput sequencing technologies and bioinformatics tools allow for whole genome comparisons and phylogenomic analyses among fungal taxa, potentially replacing molecular phylogenetic analyses based on a few marker genes shortly (Marian et al., 2024). In our research, nuclear ITS rDNA sequences were utilized for the molecular identification of ANK Akata 8687. This approach revealed a similarity of over 99% between the specimen (GenBank ID: PP494209.1) and *I. costinitii*. (Figure 7).

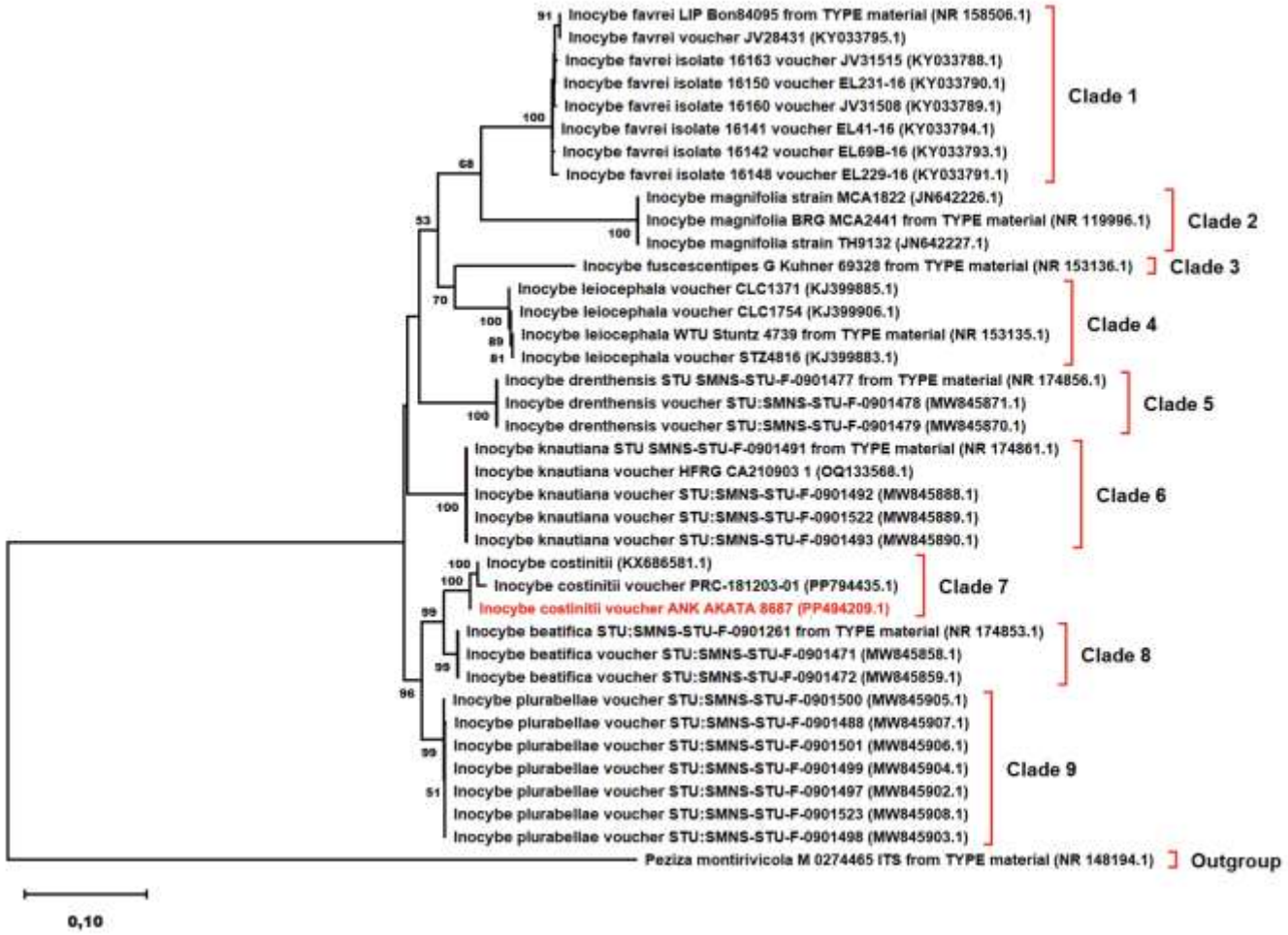


Figure **7.** The evolutionary relationships among 38 fungal specimens were depicted through a phylogenetic tree constructed using the nrITS rDNA region and the maximum likelihood (ML) method. Bootstrap rates (≥50) are assigned to each branch to indicate confidence levels. The sequences utilized in tree construction were sourced from the NCBI GenBank, except for ANK Akata 8687. Moreover, *Peziza montirivicola* was incorporated into the phylogenetic tree as the outgroup representative. GenBank accession numbers accompany each sequence, and a scale bar in the lower left corner represents a genetic distance of 0.10.

*Şekil 7. 38 mantar örneği arasındaki evrimsel ilişkiler, nrITS rDNA bölgesi ve maksimum olabilirlik (ML) yöntemi kullanılarak oluşturulan bir filogenetik ağaç üzerinde tasvir edilmiştir. Güven seviyelerini göstermek için her bir dalın yanında en az %50'lik önyükleme oranları belirtildi. ANK Akata 8687 dışında kalan ve ağaç oluşturulurken kullanılan diziler NCBI GenBank'ten alındı. Ayrıca, Peziza montirivicola, filogenetik ağaçta dış grup temsilcisi olarak dahil edildi. Her dizinin yanında GenBank erişim numaraları bulunmaktadır. Sol alt köşede bulunan genetik mesafe ölçeği, 0.10 genetik uzaklığı temsil eder.*

*Inocybe costinitii* is distinguishable on a larger scale by its moderately sized basidiomata, which feature pileus of a beige-ocher hue, enveloped in a dense, white veil. The stipe is smooth, whitish, and exhibits a swollen base. This species emits a spermatic odor and thrives in sandy, grassy areas adjacent to *Pinus halepensis* Mill. during the winter months. On a microscopic level, it is identified by its subamygdaliform spores that feature a conical to subpapillate tip, along with its fusiform cystidia (Bizio et al., 2016).

Bizio et al. (2016) described the caulocystidia as clavate to sub-fusiform or sub-ovoid, characterized by thin walls and scarcity or complete absence of apical crystals, predominantly found at the stipe's apex. In contrast, the caulocystidia observed in Turkish specimens were noted to vary from clavate to fusiform, with occasional ventricose shapes and thick walls. While some were smooth, others exhibited crystalline formations at the apex. urticoid-like structures were seldom observed.

*Inocybe costinitii* shares similar morphological and ecological characteristics with *I. griseotarda* Poirier

and *I. griseovelata* Kühner (Bandini & Huijser, 2017; Sesli, 2019; Akata et al., 2023). Distinguished by its considerable size and sturdy form, *I. griseotarda* features a greyish-white veil, with its stem initially presenting a waxy white appearance before adopting a coloration akin to the cap, predominantly covered in fine white frost-like particles. This species typically forms extensive groups during winter, thriving in symbiosis with pine and strawberry (Bizio et al., 2017). Despite these similarities, *I. griseotarda* sets itself apart with a more pronounced robustness, a fibrously cracked pileus around the center, a quickly disappearing veil, and a stem entirely dusted with fine particles and displays pinkish tones at the top. It also features spores that are longer and slimmer, pleurocystidia with thin walls, and the presence of caulocystidia not just at the apex of the stipe but along its entire length (Bandini & Huijser, 2017; Bizio et al., 2016; 2017). *I. griseovelata* is characterized by its distinctive velipellis, which is whitish to grayish and densely covers the pileus. The surface of the pileus is generally smooth, though it may exhibit some innate fibrils. The stipe features light powdery deposits limited to the uppermost part. This species is recognized for its relatively large spores and typically has elongated hymenial cystidia with broad necks. Moreover, it exhibits long and slender caulocystidia. It is frequently found in areas with calcareous soil and can be associated with broad-leaved species and conifers (Akata et al., 2023; Bandini et al., 2022). This species diverges from *I. costinitii* due to its darker pileus, a veil of beige-grey coloration, a uniform thick stipe, and a pruinose apex. The lower part of the stipe is adorned with greyish fibrils. Moreover, it is characterized by its distinct subcylindrical cystidia (Bizio et al., 2016).

In the current study, *Inocybe costinitii* has been identified and documented from Türkiye for the first time, adding to the diversity of Turkish *Inocybe*. This report increases the number of *Inocybe* species known in the country's border to 82.

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## Contribution of The Authors As Summary

The authors declare that their contributions are equal.

## Statement of Conflict Of Interest

The authors have declared no conflict of interest.

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